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OVIPOSITION SITE SELECTION BY MOSQUITOES: PREDATOR-RELEASED
KAIROMONES AND RESPONSES TO DIFFERENT TAXA

A Thesis
submitted in partial fulfillment of requirements
for the degree of Master of Science
in the Department of Biology
The University of Mississippi

By
LAUREN L. EVELAND

May 2015

ABSTRACT

Oviposition site selection is a critical reproductive behavior for egg-laying organisms that offer little to no parental care. Determining the mechanisms females use to assess sites is critical to understanding life history traits, community structure, and species interactions. Mosquitoes are excellent model organisms for studying oviposition site selection for numerous reasons: 1) a poor decision can lead to zero reproductive success, 2) females actively assess and choose among habitat patches when locating an oviposition site, 3) larvae remain in the habitat where they hatch until they metamorphose, and 4) they lay conspicuous egg rafts that allow direct assessment of oviposition. Certain mosquito species actively detect and avoid habitats with predaceous backswimmers (Heteroptera: Notonectidae), and certain species of fish, including *Gambusia affinis*. For my thesis I focused on mosquitoes of the genus *Culex*, and how they responded solely to the presence of kairomones (chemicals emitted by an organism and detected by an individual of another species; where only the receiver benefits from the cue) released by *Gambusia affinis* (Western mosquitofish), *Lepomis cyanellus* (green sunfish), and *Procambarus hayi* (straightedge crayfish) when locating oviposition sites. Three paired experiments (Control vs. Predator-conditioned water) were conducted, one for each predator species. A fourth experiment was conducted to determine where *Culex* would oviposit in the presence of positive, negative, and neutral stimuli. Oviposition choice was quantified by collecting all egg rafts laid in experimental pools. Egg rafts were hatched, raised, and identified to species. In experiment one, *Culex restuans* used kairomones to detect and avoid ovipositing in the presence of *G. affinis*. In experiment two, *L. cyanellus* kairomones did not deter *Culex* species.

In experiment three, mosquitoes were attracted to pools with crayfish-conditioned water over Controls. In experiment four, presence of *G. affinis* within a pair reduced the number of egg rafts found in Control and Crayfish pools. When not paired with *G. affinis*, Control and Crayfish pools received significantly more egg rafts than *G. affinis* pools. Overall, kairomones alone can drive oviposition site choice in *Culex restuans*, and *Culex* perceive quality habitats as low quality when in close proximity to *G. affinis* pools.

LIST OF ABBREVIATIONS

Culex p×q: Introgressed hybrid populations of *Culex pipiens* and *Culex quinquefasciatus*

PCR: Polymerase chain reaction

ANOVA: Analysis of Variance

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Through my graduate school experience, I have met many wonderful and interesting people at both Texas Tech University and The University of Mississippi. In particular, I would like to thank my committee members at TTU: Dr. William Resetarits, Dr. Kenneth Schmidt, Dr. Ximena Bernal, and Dr. Kerry Griffis-Kyle, and at UM: Dr. William Resetarits, Dr. Paul Lago, and Dr. Jason Hoeksema. There are more, too many to list, so I will just thank you all for your support and knowledge. I would especially like to thank Dr. William Resetarits, my graduate advisor, for giving me the opportunity to work in his lab. Matt Pintar, Jason Bohenek, Bob

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INTRODUCTION

Selecting a habitat is an important process that is mediated by organisms in response to both abiotic and biotic factors. Oviposition site selection is a particularly critical behavior since it ultimately determines the welfare and survival of offspring as well as the fitness of the parents (Resetarits and Wilbur 1989). Organisms that offer little to no parental care must actively choose oviposition sites of high quality considering both abiotic and biotic factors.

In particular, I was interested in determining how mosquitoes choose oviposition sites. I investigated this idea by asking three questions: 1) Can mosquitoes from the genus *Culex* detect kairomones released by fish predators when selecting an oviposition site, 2) Do mosquitoes detect the presence of crayfish via kairomones when locating an oviposition site, 3) What is the distribution of egg rafts when mosquitoes are given choices among positive, negative, and control stimuli, and is there context-dependence in oviposition site choice. I answered these questions by conducting four field mesocosm experiments with natural populations of *Culex* mosquitoes.

CHAPTER I: DETECTION OF PREDATORY FISH KAIROMONES BY OVIPOSITING MOSQUITOES

I.I INTRODUCTION

Selecting an oviposition site is a reproductive behavior where individuals actively choose the location in which they lay their eggs (Rausher 1983, Singer 1984). This behavior is critical for organisms such as amphibians and aquatic insects that possess complex life cycles, lay eggs in aquatic habitats, have a short life-span, and often reproduce only once in their lifetime. The only parental care offered by most of these animals is selecting a habitat in which to oviposit. Therefore, it is important for females to choose a safe and resource-rich habitat for their offspring (Resetarits and Wilbur 1989). Parental fitness is dependent upon the survival and success of offspring; therefore it is crucial a female selects a location with abiotic and biotic factors that maximize expected fitness (Resetarits and Wilbur 1989, Resetarits 1996, Blaustein et al. 2004, Hocking and Semlitsch 2008). Since choosing a high quality oviposition site is so important to fitness, it should be subject to strong selection (Resetarits 1996).

Studies examining oviposition site selection in aquatic systems have been conducted for a variety of aquatic insects (Chesson 1984, Petranka and Fakhoury 1991, Blaustein and Kotler 1993, Berendonk 1999, Resetarits 2001, Binckley and Resetarits 2005) and amphibians (Resetarits and Wilbur 1989, Crump 1991, Kats and Sih 1992, Binckley and Resetarits 2002, Rieger et al. 2004). Identifying the mechanisms animals use to locate oviposition sites is important for researchers to better understand how organisms evaluate habitats and how those

choices impact community structure and species interactions. The research I conducted provides insight into how and where *Culex* mosquitoes choose to oviposit when predators are present and the precise sensory mechanism used to assess habitats.

Using semiochemicals, particularly kairomones, is one mechanism that studies have suggested is involved in predator detection by ovipositing mosquitoes (Chesson 1984, Petranka and Fakhoury 1991, Angelon and Petranka 2002). Kairomones are chemicals emitted by an organism and detected by an individual of another species; in this interaction only the receiver benefits from the cue (Brown et al. 1970). Mosquitoes from the genus *Culex* and *Culiseta* can detect predatory backswimmers, *Notonecta* species (Heteroptera: Notonectidae), via kairomones (Eitam and Blaustein 2004, Blaustein et al. 2005, Silberbush and Blaustein 2008), but the mechanism for fish detection has not yet been definitively determined. Previous studies suggested that mosquitoes may use kairomones to detect fish, but these studies either, 1) used caged fish (Petranka and Fakhoury 1991, Hurst et al. 2010), which can still produce visual and mechanical cues (Berendonk 1999), 2) did not collect egg rafts (Angelon and Petranka 2002), which means oviposition and female choice was not directly quantified, 3) conducted only lab experiments (Van Dam and Walton 2008), or 4) used different species of mosquitoes that did not respond to chemical cues (Walton et al. 2009).

Mosquitoes are excellent model organisms for conducting oviposition site selection studies because they meet several important criteria: 1) a poor decision can lead to zero reproductive success, 2) they actively assess and choose among habitat patches when locating an oviposition site, 3) larvae remain in the habitat where they hatch until they undergo metamorphosis, and 4) they lay conspicuous egg rafts that allow direct assessment of oviposition (Rausher 1983, Singer 1984, Resetarits and Wilbur 1989, Resetarits 1996, Blaustein et al. 2004).

Mosquitoes themselves are of interest in several contexts. Mosquitoes are important to humans since many species are disease vectors. In particular, the species I studied, *Culex restuans* Theobald and *C. p×q* (see Methods) can carry St. Louis encephalitis and West Nile virus (Hamer et al. 2008, Turell 2012). Thus, examining where mosquitoes prefer to oviposit can potentially aid in controlling their populations. Also, mosquitoes are important food items for a variety of both terrestrial and aquatic animals that feed on every life stage of mosquito ranging from eggs to adults. Thus, there are contrasting and complex effects of mosquitoes on humans and other animals. Therefore, it is important to determine the impact of mosquitoes on community structure and species interactions.

In this context I asked the question: Can mosquitoes of the genus *Culex* use kairomones alone to detect two different fish species that are predators of larval mosquitoes (*Gambusia affinis*, Western Mosquitofish, and *Lepomis cyanellus*, green sunfish) when selecting an oviposition site? I hypothesized that female mosquitoes would be selective in where they laid their eggs, and predicted they would detect fish-released kairomones and avoid ovipositing in these habitats conditioned with only fish chemicals. To answer the initial question, I conducted two field experiments with artificial pools to examine oviposition in natural populations of *Culex* mosquitoes.

I.II METHODS

I.II.I Study Site

I conducted two experiments at Tyson Research Center (795.8 hectares) of Washington University, St. Louis County, MO (38.5167°N, 90.5500°W). Tyson is located along the

Meramec River in the Ozark Border region. The habitat consists of primarily hardwood oak and hickory secondary forests along with maple, sycamore, and cottonwood in the bottomland. Throughout Tyson there are patches of old fields, and scattered ephemeral and permanent ponds and seasonal creeks.

I.II.II Description of Study Organism

I.II.III Mosquitoes

Culex mosquitoes were my focus since they are abundant at Tyson. In addition, they lay their eggs in rafts, which are easier to collect and quantify than individually laid eggs. Each egg raft collected represents one mosquito's choice of an oviposition site. The two most abundant *Culex* species were *C. restuans* and *C. p×q*. The latter is a widespread, introgressed population of hybrid origin, derived from *C. pipiens* Linnaeus and *C. quinquefasciatus* Say (Barr 1957, Harbach 2012). A large sample of larvae from the same site was identified using PCR as *C. pipiens* × *C. quinquefasciatus* hybrids (Silberbush and Resetarits *unpublished data*). These two species are known vectors of West Nile Virus (Hamer et al. 2008, Turell 2012).

I.II.IV Fish Predators

Gambusia affinis (Baird and Girard) (~ 1g), and *L. cyanellus* (Rafinesque) (~ 7-11g), were the predators used in this experiment. These species of fish were selected since they are native to Missouri and are predators of mosquito larvae (DuRant and Hopkins 2008, Silberbush and Resetarits *in review A*). *Lepomis cyanellus* also prey on mosquito egg rafts (*personal observation*). Fish were collected from ponds located at Tyson and placed into separate 1,211 L outside holding tanks. Eight individuals of each species were removed haphazardly from the

outside holding tanks and each species was placed into separate 10 gallon glass aquaria ($50.8 \times 27.9 \times 30.5$ cm) in the lab for two days. Fish were fed fish flakes (TetraMin® Tropical flakes - Tetra Holding inc., Germany) the first day and then gut cleared (not fed) for twenty-four hours before use in the experiments. This was done to eliminate the chance of the fish consuming mosquitoes, which could lead to conspecific detection by ovipositing mosquitoes during the study. Mosquitoes can detect conspecifics from fish excrement which could interfere with the predator-released kairomones (Kats and Dill 1998).

I.II.V Experimental Design

Two artificial pool experiments were conducted simultaneously from 29 July 2013 - 14 August 2013. Both consisted of the same experimental design and collection method. The experiments were separated spatially by 152 m and differed only in the species of fish used as the predator (*G. affinis* or *L. cyanellus*). The two sites were old fields with relatively open canopy. The arrays consisted of 16 black plastic tubs ($66 \times 45 \times 16$ cm), hereafter called pools, separated into eight pairs. Experimental pools were placed one meter apart (Fig.1) while each pair was three meters from neighboring pools. All pools were filled with treated well-water and left for two days to age and to insure chlorine volatilized from the water. Ten grams of rabbit chow (Small World Rabbit Food - Mannapro, St. Louis, MO; 40% protein) was added to each pool to promote detection by mosquitoes.

I used one predator species in each of the two arrays, either *G. affinis* or *L. cyanellus*. At the start of the experiment, one pool in each pair (eight pairs in two sites) was randomly assigned as the predator treatment. One fish was haphazardly assigned to a Fish treatment pool and paired with a Control (no fish) pool. Each of the pools contained a cage consisting of a black plastic

plant pot (32 cm diameter) with two screened sides (1mm mesh) and a screen lid. The fish were placed inside the cages and left in the pools for two and half days (57 hours) to condition the water with fish chemicals. The screen, on the cages, allowed for the fish chemicals to disperse through the water. The pools were covered to ensure that colonization by other insects and mosquito oviposition did not occur before the start of the experiment. Before dusk on day three fish were removed, and pools were uncovered to allow for mosquito oviposition. This process was repeated four times using different fish for each iteration. The same pools and water were used throughout the experiment. The first collection day in the *G. affinis* array was 3 August 2013, and 5 August 2013 for the *L. cyanellus* array. Each morning all egg rafts were collected and taken to the lab. Once the eggs hatched they were reared to fourth instar and identified to species following Darsie and Ward (2005).

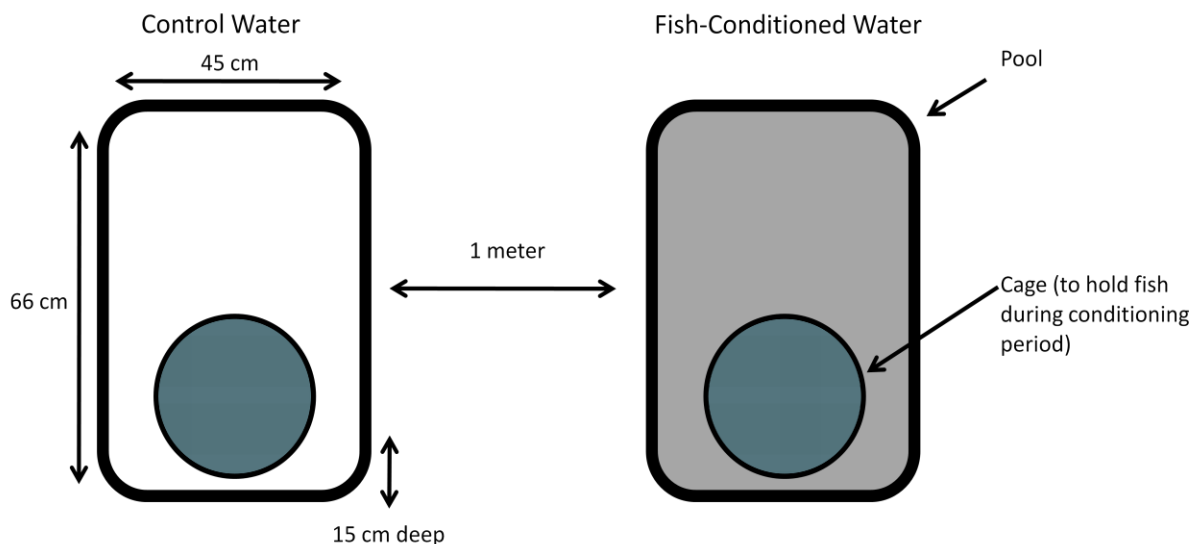


Fig. 1. Experimental setup for Control versus Fish-conditioned water. Experimental pools were paired with each containing a cage. The white pool represents a Control and the gray pool represents Fish-conditioned water. Fish treatments were conditioned with fish while the pools were covered. Oviposition was allowed only after the fish were removed from pools.

I.II.VI Data Analysis

For both experiments (Experiment 1 and 2 analyzed separately), I calculated the mean number of egg rafts collected per pool per day (daily average). The data were square-root transformed [$\sqrt{(x + 0.5)}$; (Yamamura 1999)] and analyzed with a paired, one-tailed Student's t-test to determine whether the mean number of egg rafts in the Control pools differed from the mean number in Fish treatments. A one-tailed test was conducted since there is evidence from previous studies that mosquitoes avoid certain fish species. All statistical analyses were performed in R Studio 0.98.994 with $\alpha = 0.05$.

I.III RESULTS

Across both experiments, we collected 1261 egg rafts: 72.8% *Culex restuans* and 25.9% *C. p×q*. Other species were not analyzed due to very low abundance (1.3% of total).

In the *G. affinis* experiment, a total of 321 egg rafts were collected: 238 (74.1%) *C. restuans* and 83 (25.8%) *C. p×q*. *Culex restuans* laid 206 (87%) (6.43 ± 1.63 , mean ± 1 SE) egg rafts in the Control pools and 32 (13%) (1.00 ± 0.37 , mean ± 1 SE) egg rafts in the *G. affinis* pools. *Culex p×q* laid 51 (61%) (0.91 ± 0.32 , mean ± 1 SE) egg rafts in the Control pools and 32 (39%) (0.57 ± 0.11 , mean ± 1 SE) egg rafts in *G. affinis* pools. There was a significant effect of *G. affinis*-conditioned water on *C. restuans* with the mean number of egg rafts greater in the Controls ($t = 4.00$, $df = 7$, $p\text{-value} = 0.0026$), but they had no effect on *C. p×q* ($t = 0.71$, $df = 7$, $p\text{-value} = 0.2505$, Fig. 2A).

In the *L. cyanellus* experiment, there were two instances of fish mortality, and those fish were replaced as soon as they were discovered (the morning after the fish were placed into the

pools). A total of 940 egg rafts were collected: 692 (73.6%) *C. restuans* and 248 (26.4%) *C. p×q*. *Culex restuans* laid 411 (59%) (12.84 ± 3.12 , mean ± 1 SE) egg rafts in the Control pools and 281 (41%) (8.78 ± 1.21 , mean ± 1 SE) egg rafts in the *L. cyanellus* pools. *Culex p×q* laid 128 (52%) (4.00 ± 1.09 , mean ± 1 SE) egg rafts in the Control pools and 120 (48%) (3.75 ± 0.50 , mean ± 1 SE) egg rafts in the *L. cyanellus* pools. *Lepomis cyanellus*-conditioned water did not have a significant effect on oviposition of either *Culex* species, *C. restuans* ($t = 1.15$, $df = 7$, $p\text{-value}=0.1447$) or *C. p×q* ($t = 0.20$, $df = 7$, $p\text{-value}= 0.4241$) (Fig. 2B).

I. IV DISCUSSION

Selecting an oviposition site is an extremely important aspect of female mosquito behavior since it represents the only apparent parental care. Detection and avoidance of predators should increase offspring performance and survival. Overall, oviposition behavior can affect species distribution, species interactions, and community structure (Resetarits and Wilbur 1989). Thus, it is important to understand the mechanisms mosquitoes use to detect and avoid predators, and what predators they choose to avoid, when selecting oviposition sites. The focus of my research was to determine whether or not *Culex* mosquitoes use fish-released kairomones to detect the presence of predatory fish.

My results indicate that female *C. restuans* use kairomones to detect and avoid *G. affinis* when selecting an oviposition site. However, they did not avoid pools conditioned with *L. cyanellus* chemical cues, despite *L. cyanellus* having 7-10x higher biomass than *G. affinis*. However, *C. p×q* was not deterred by kairomones of either fish species. While both fish species are effective predators of mosquito larvae (DuRant and Hopkins 2008, Resetarits and Silberbush *in review A*) the two mosquito species respond differently to the fish-released kairomones.

Numerous factors could lead to the differential responses, 1) the fish species may feed in different microhabitats; *G. affinis* usually forage at the water surface while *L. cyanellus* tend to forage throughout the water column. Mosquito larvae and egg rafts are normally located at the water surface, 2) *G. affinis* are small even as adults while *L. cyanellus* become larger as adults leading to a possible change in prey preference, 3) *G. affinis* can consume up to 1,000 mosquito larvae in a 24-hour period (DuRant and Hopkins 2008); therefore, large numbers of individuals would be very successful at consuming high numbers of mosquito larvae within a single habitat. *Lepomis cyanellus* may not have the same effect on mosquito larvae, 4) *L. cyanellus* may possess a chemical camouflage. A form of chemical camouflage has been observed in another species of freshwater fish, *Aphredoderus sayanus* (Gilliams) (pirate perch) (Resetarits and Binckley 2013, Silberbush and Resetarits *in prep*). *Aphredoderus sayanus* are effective predators of both frog and mosquito larvae and aquatic beetles, but do not deter ovipositing *Culex* species, frogs, or colonizing beetles (Resetarits and Binckley 2013, Silberbush and Resetarits *in review A*). Additional research is needed to determine the reason why *Culex* mosquitoes are not deterred by certain species of fish that prey on their eggs and larvae.

Both *C. restuans* and *C. p×q* have similar ecological niches, but their oviposition behavior differs in the presence of fish-released kairomones. While *C. restuans* avoided *G. affinis*, *C. p×q* was not deterred by them. This finding was not expected since *C. p×q* meets the requirements for evolving selective oviposition behavior (Resetarits and Wilbur 1989, Resetarits 1996, Blaustein 1999). My results are congruous with previous findings concerning *Culex quinquefasciatus* Say, which does not respond to *G. affinis* chemical cues (Walton et al. 2009), as well as *C. p×q* displaying limited sensitivity to *G. affinis* kairomones (Angelon and Petranksa 2002). In contrast, Resetarits and Silberbush (*in review B*) found evidence that *C. p×q* do in fact

avoid *G. affinis*. The only difference between the two experiments was the continued presence of one caged, *G. affinis* while I tested only chemical cues. An explanation for this behavior that may also explain the different responses of *C. restuans* to certain fish species is that *C. p×q* may use multiple cues (visual, mechanical, auditory, and/or chemosensory) to detect predators. A second explanation could be that the larvae of the two *Culex* species behave differently in the presence of predators. *Culex p×q* larvae could be less active than *C. restuans* larvae therefore drawing less attention from predators. Kesavaraju et al. (2011) demonstrated that *Culex pipiens* larvae were less active than the other larval mosquitoes examined. They found that *C. pipiens* spent most of their time at the surface of the water motionless.

The detection of fish-released kairomones is a possible adaptation to decrease the offspring mortality. Numerous lab based studies discovered that other aquatic insects respond to chemicals of certain fish predators, 1) the mosquito *C. tarsalis* Coquillett, laid significantly fewer eggs in treatments conditioned with *G. affinis* chemicals (Van Dam and Walton 2008), the mosquito *C. annulirostris* Skuse, selected against ovipositing in water conditioned with the *Melanotaenia duboulayi* (Castelnau) (crimson-spotted rainbowfish) (Hurst et al. 2010), and 3) two species of phantom midges, *Chaoborus crystallinus* De Haan and *C. obscuripes* (van der Wulp), laid significantly fewer eggs in treatments conditioned with chemical cues from *Scardinius erythrophthalmus* (Linnaeus) (common rudd) and *Pungitius pungitius* (Linnaeus) (sticklebacks) (Berendonk 1999). A recent field study found that *Culiseta longiareolata* Macquart used predator-released kairomones to detect *Notonecta maculata* Fabricius when locating an oviposition site (Silberbush et al. 2010). Although there has been evidence that *Culex* mosquitoes use fish-released kairomones, my study demonstrated that natural populations of

Culex restuans use kairomones to detect the presence of *G. affinis*. Further studies should be conducted to better understand why mosquito species respond differently to certain fish species.

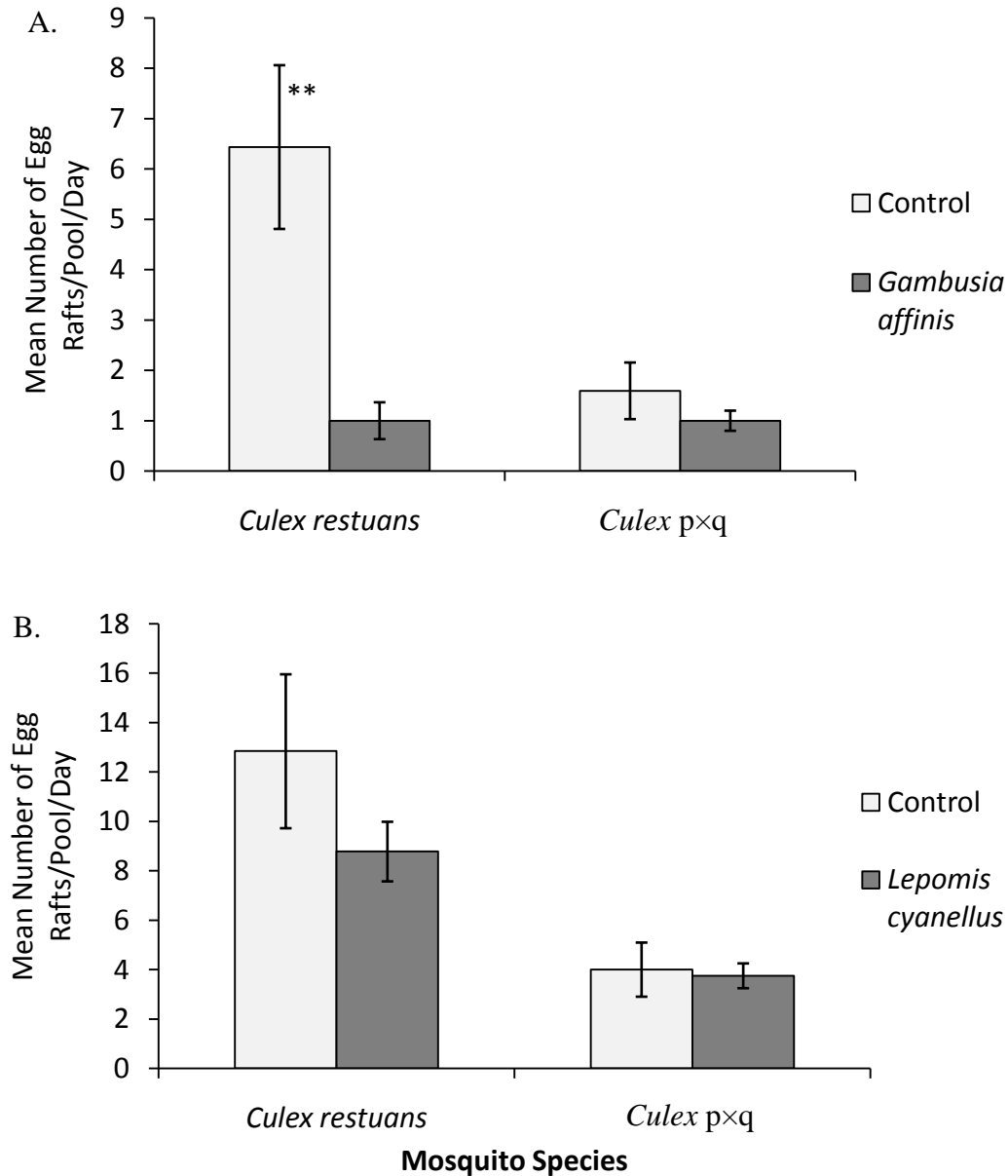


Fig. 2. Mean number (± 1 SE) of egg rafts/pool/day in Control and Fish-released kairomone treatments. The white bars represent Controls and the grey bars represent Fish treatments including (A) *Gambusia affinis* and (B) *Lepomis cyanellus*: paired, one-tailed Student's t-test, *, $p < 0.05$, and **, $p < 0.01$.

CHAPTER II: CONTEXT DEPENDENT OVIPOSITION SITE SELECTION IN *CULEX* MOSQUITOES: EFFECTS OF POSITIVE, NEGATIVE, AND NEUTRAL STIMULI

II.I INTRODUCTION

Certain species of mosquitoes have been found to avoid predator-released kairomones from both predatory backswimmers (Heteroptera: Notonectidae) (Silberbush et al. 2010) and certain fish species (Walton 2008, Eveland et al. *submitted*). However, little is known regarding how ovipositing mosquitoes respond to chemical cues from other (non-fish or Notonecta) predators.

Crayfish are opportunistic omnivores (Butler and Stein 1985, Crandall and Buhay 2008) that feed on detritus, vegetation, invertebrates, crustaceans, and small vertebrates. Adult and juvenile crayfish tend to have different diets: adult crayfish feed mostly on detritus and vegetation while juveniles are mostly carnivorous (Butler and Stein 1985, Goddard 1988). Mkoji et al. (1999) found that adult invasive *Procambarus clarkii* (Girard) (Louisiana red swamp crawfish) consumed mosquito larvae when alternative food was not available, while juveniles preferred mosquito larvae even when alternative food was present.

I am interested in how ovipositing mosquitoes respond to kairomones released by possible larval predators from taxa other than fish and aquatic insects, specifically crayfish dwelling in lotic-ephemeral water bodies. I hypothesized that gravid mosquitoes would be

selective when choosing a site to oviposit. For this study I asked two questions. First, I examined how *Culex* mosquitoes respond to the presence of crayfish-released kairomones when selecting an oviposition site. I predicted that mosquitoes would avoid ovipositing in pools conditioned with crayfish-released kairomones. Crayfish are known predators of mosquito larvae so it would be advantageous for mosquitoes to avoid laying their eggs in habitats where they are present. The results from experiment one demonstrated that crayfish-released kairomones attracted gravid mosquitoes and therefore crayfish chemicals seem to be a positive stimulus. Based on the results from the first experiment, I examined where *Culex* mosquitoes deposit their eggs when given paired choices involving positive, negative, and neutral stimuli.

II.II METHODS

II.II.I Mosquito oviposition in the presence of crayfish-released kairomones

II.II.I.I Study Site

This experiment was conducted at the University of Mississippi Field Station (299.5 hectares), Lafayette County, MS (34.2557°N, 89.2326°W) from 2 - 9 June 2014. The field station is located within the Eocene Hills in the Interior Coastal Plain. It contains both natural and constructed wetlands, springs, streams, grassland, and forest. There are approximately 200 experimental ponds that are fed by natural springs. The forest is mixed shortleaf pine and oak along with loblolly pine, sweet gum, black gum, winged elm, and red maple.

II.II.I.II Study Organism

For this experiment, I examined oviposition site choice in *Culex* mosquitoes. Species of *Culex* are abundant at the field site. In addition, they lay their eggs in rafts, which are easier to

collect and quantify than individually laid eggs. Each egg raft represents one mosquito's choice in an oviposition site. For the above reasons, mosquitoes from the genus *Culex* were the focal organisms for this study.

II.II.I.III Predator used for conditioning treatment

Procambarus hayi (Faxon) (Straightedge crayfish) were used as the predator in this experiment. This crayfish was selected since they are native to Mississippi, are opportunistic foragers, and will prey on mosquito larvae (Mkoji et al. 1999 and *personal observation*). The crayfish used in this experiment (n=20) were collected from ponds at the University of Mississippi Field Station. Crayfish were caught using minnow traps baited with dog chow and dip nets. The collection of crayfish was authorized under permit number 0624143, distributed by Mississippi Department of Wildlife, Fisheries, and Parks.

II.II.I.IV Experimental Design

The study was designed as a paired test with a Control and a Crayfish treatment consisting of crayfish-conditioned water. I set up a total of 16 black tubs ($66 \times 45 \times 16$ cm), hereafter called pools, with eight pairs separated spatially by at least 12 m. Each pool within a pair was separated by $\frac{1}{2}$ m and pairs were 3 m from the forest edge (Fig. 3). On day one of the experiment, 15 L of well-water and 15 g of rabbit chow were added to all of the experimental pools. In addition, 5 L of crayfish-conditioned water was added to Crayfish treatments, and 5L of control water was added to the Controls for a total of 20 L of water in all pools. Before dusk on nights two thru six, 5 L of water was removed from all experimental pools, and 5 L of fresh crayfish-conditioned or control water was added back to all of the pools (Fig. 4). This allowed

for the chemical cues to stay fresh in the crayfish-conditioned pools, and for the water volume to remain constant throughout the experiment. For a total of eight days, water was removed from the conditioning pool or control pool and added to the experimental pools. The amount of water removed from the conditioning pool and control pool was replenished from the tanks holding aged pond water in order to have enough water for the next day. Each morning, all egg rafts were collected from the experimental pools, for a total of seven collection days, and taken back to the lab. Once the eggs hatched, larvae were raised to fourth instar and identified to species following Darsie and Ward (2005).

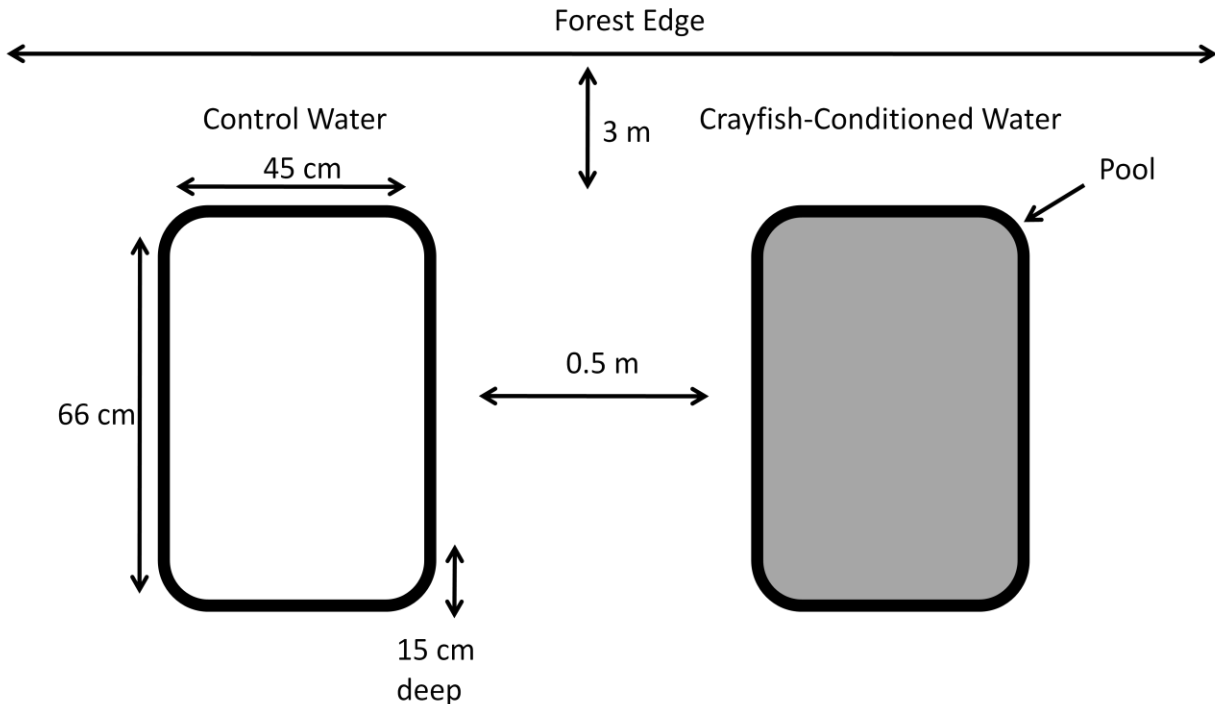


Fig. 3. Experimental setup for Control versus Crayfish-conditioned water. Experimental pools were in 8 pairs separated spatially through the field site. The white pool represents the Control and the gray pool represents Crayfish-conditioned water.

II.II.I.V Water Conditioning Process

The water conditioning process was similar to the one used by Angelon and Petranka (2002). Crayfish were kept separately in 15 L plastic containers ($42.5 \times 30.2 \times 17.8$) with air stones and fed fish flakes (TetraMin® Tropical flakes -Tetra Holding inc., Germany) and algae pellets. Before the crayfish were placed in the conditioning pool they were allowed to clear their gut (not fed) for 24-hours. The gut clearing process took place in plastic containers with fresh well-water and no food for the crayfish to consume. Each crayfish was also massed before being added to the conditioning pool (4.44 ± 1.47 g, mean \pm SD); a small blue pool (1 m diameter) was used as the conditioning pool and was filled with 60 L of pond water. The pool was placed in the grass under a tree to ensure the crayfish were not in direct sun. The pond water was left to sit for two weeks and covered to insure no organisms colonized or oviposited in the water. Pond water was used for both the treatment and control pools. Twenty crayfish were added to the conditioning pool and remained there for the duration of the experiment (eight days). The number of crayfish used is based on the density of predators used in prior studies (Angelon and Petranka 2002 and Walton et al. 2009).

II.II.I.VI Data Analysis

The mean number of egg rafts per experimental pool was compared between Control pools and Crayfish-conditioned pools. Data was square-root transformed [$\sqrt{(x + 0.5)}$; (Yamamura 1999)] in order to obtain normality and analyzed using a paired, two-tailed Student's t-test in the statistical program R v 3.0.2 (R 2013) with $\alpha = 0.05$.

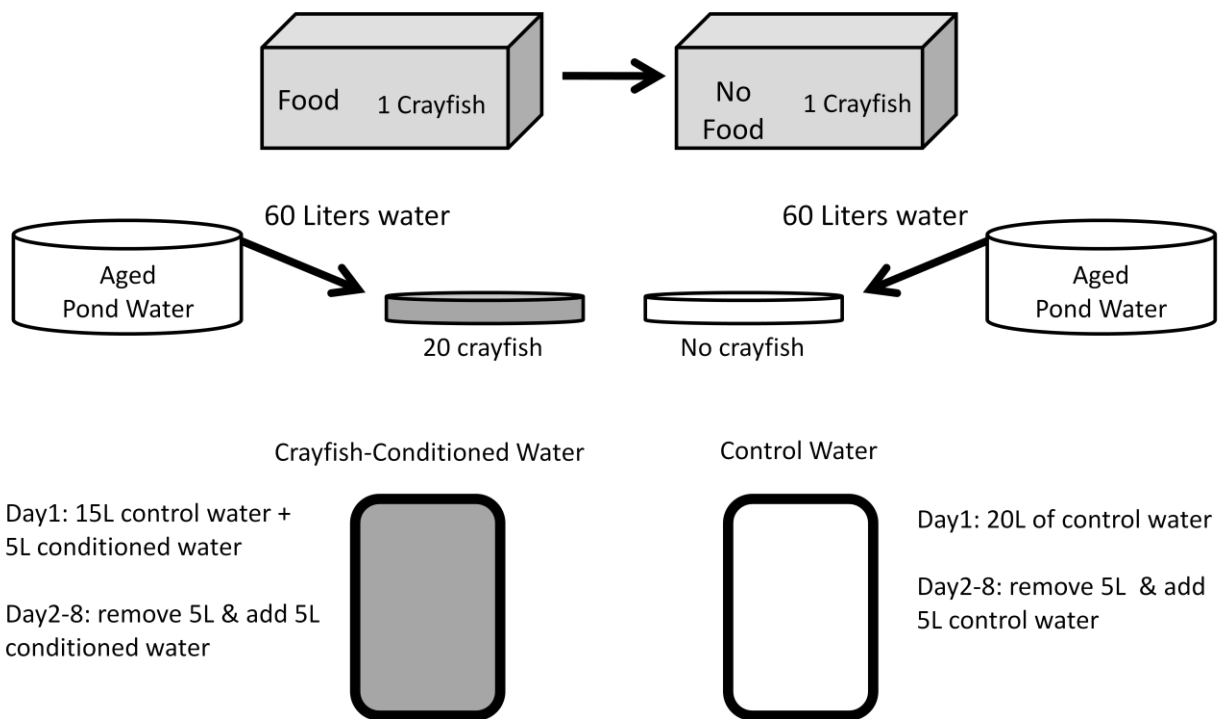


Fig. 4. Water conditioning process for making crayfish-conditioned water. Crayfish were held in the lab up to 48 hours before being moved outside to 1.5 m plastic pools in order to condition water for the Crayfish-conditioned pools. Twenty adult crayfish were added to the plastic pools for 24 hours before the water was used in the experimental pools.

II.II.II Oviposition site selection in the presence of positive, negative, and neutral stimuli

II.II.II.I Study Site

This experiment was also conducted at the University of Mississippi Field Station, Lafayette County, Mississippi. The experiment ran from 11 August 2014 - 25 September 2014.

II.II.II.II Predator Description

The *P. hayi* (straightedge crayfish) was used as the positive stimuli in this experiment. Results from the previous experiment concluded that *P. hayi* have an attractant effect on *Culex* mosquitoes. Crayfish (n=18) were housed individually in 15 L plastic containers (42.5 × 30.2 × 17.8) and fed fish flakes until they were utilized in the experiment.

Gambusia affinis were used as the negative stimuli in this experiment because they are native to Mississippi and known to deter certain *Culex* mosquitoes. All fish (n=18) were collected from ponds located at the University of Mississippi Field Station with dip nets and minnow traps. Fish were housed individually in 15 L plastic containers ($42.5 \times 30.2 \times 17.8$) and fed fish flakes until they were utilized in the experiment. The collection of fish was authorized under permit number 0624143, distributed by Mississippi Department of Wildlife, Fisheries, and Parks.

II.II.III Experimental Design

This experiment consisted of nine blocks located at three sites and conducted at three different times with three blocks running simultaneously. Each block contained three pairs of black tubs ($66 \times 45 \times 16$ cm), hereafter called pools. The treatments for each pair of pools were randomly assigned as follows: 1) Control and *P. hayi*, 2) Control and *G. affinis*, and 3) *P. affinis* and *G. affinis*. The position of each pair was randomized for each block. Each pool within a pair was separated by 1 m, each pair was 10 m apart, and all pools were placed 3 m from the forest edge. For the first six blocks, a cage consisting of a black plastic pot (32 cm diameter) with two screened sides (1mm mesh) and a screened lid was placed in each of the pools to insure the predators could not consume mosquito egg rafts or larvae while still allowing chemical cues to diffuse through the water. In blocks seven through nine, the pools were covered with 1mm mesh screen sunk into the water instead of using a cage. This is another technique to separate the predators from the mosquitoes but still allowing predator-released kairomones to diffuse through the water. The pools were filled with well-water, 50 g of homogenized leaf litter as a nutrient source, and two limestone rocks (~7.6 cm and 15.2 cm in diameter). The rocks were used to

buffer the water, and to keep the cages in place. The crayfish and fish were massed (crayfish: 5.96 ± 2.31 g, fish: 0.67 ± 0.38 g, mean \pm SD) and randomly assigned to pools in each block. The experimental pools were opened to the indigenous mosquito population for them to lay eggs at will. The experiment ran for ten days, at which time the experiment was dismantled and re-established as above. This process continued until a total of nine blocks was completed. Egg rafts were collected each morning for 10 days, taken back to the lab, hatched, raised to fourth instar, and identified to species following Darsie and Ward 2005.

II.II.IV Data Analysis

Two ANOVAs were run based on a randomized complete block design. Treatment and block were the predictor variables in both analyses. The first ANOVA compared the overall number of egg rafts in *G. affinis*, *P. hayi*, and Control pools. A second ANOVA was run to compare the total number of egg rafts in pairs of pools of different configurations: *G. affinis* and Control, *P. hayi* and Control, and *G. affinis* and *P. hayi*. For both of the ANOVAs I ran a Post Hoc Tukey's honest significance difference test to determine which treatments were different from one another. I ran three paired, two-tailed Student's t-tests to examine spatial context dependence, comparing mean number of egg rafts in focal treatments when paired with different treatments: Control pools with *G. affinis* pools vs. Control pools with *P. hayi* pools, *P. hayi* pools with Control pools vs. *P. hayi* pools with *G. affinis* pools, and *G. affinis* pools with Control pools vs. *G. affinis* pools with *P. hayi* pools. All statistical analyses were conducted in the statistical program R v 3.0.2 with $\alpha = 0.05$, and all data were square-root transformed [$\sqrt{(x + 0.5)}$] (Yamamura 1999) to obtain normality.

II.III RESULTS

II.III.I Paired oviposition test in the presence of crayfish-released kairomones

A total of 255 mosquito egg rafts were collected, all of which were identified as *C. restuans*; 80 (31%) (10.0 ± 0.39 , mean ± 1 SE) were laid in the Controls and 175 (69%) (22.0 ± 0.96 , mean ± 1 SE) in Crayfish-conditioned pools. The presence of *P. hayi* kairomones had a significant effect on where *C. restuans* oviposited ($t = -2.82$, $df = 7$, $p\text{-value} = 0.0256$) (Fig. 5), with ovipositing females preferring Crayfish-conditioned pools.

II.III.II Oviposition site choice in paired sets of positive, negative, and neutral stimuli

A total of 680 mosquito egg rafts were collected; 569 (84%) *C. restuans*, 49 (7%) three other species of *Culex*, and 62 (9%) that never hatched and were unidentifiable. The number of egg rafts collected from the three rarer species of *Culex* was not sufficient for statistical analysis.

Data were analyzed with two ANOVAs with treatment and block as the predictor variables, and three paired Student's t-tests. All data was square-root transformed [$(\sqrt{x + 0.5})$] (Yamamura 1999). The first analysis compared the total number of egg rafts deposited in the *G. affinis*, *P. hayi*, and Control pools. The number of egg rafts deposited in these treatments was significantly different ($F_{2, 16} = 10.42$, $p\text{-value} = 0.0013$) (Fig. 6), with significantly more egg rafts in the *P. hayi* and Control pools than *G. affinis* pools. The block effect was marginally significant ($F_{8, 16} = 2.48$, $p\text{-value} = 0.0577$). The second analysis compared the total number of egg rafts in pairs of pools of different configurations (*G. affinis* and Control, *P. hayi* and Control, *P. hayi* and *G. affinis*). The number of egg rafts was significantly different among the pairs of pools ($F_{2, 16} = 7.89$, $p\text{-value} = 0.0041$) (Fig. 7). There were significantly more eggs in paired *P. hayi* and Control pools than paired *G. affinis* and Control pools and paired *P. hayi* and *G. affinis*

pools. There was a block effect, with blocks being significantly different ($F_{8, 16} = 3.45$, p-value = 0.0168). For the final analysis, I ran three different paired, two-tailed Student's t-tests to compare the mean number of egg rafts found in Controls with *P. hayi* vs. Controls with *G. affinis*, *G. affinis* with Controls vs. *G. affinis* with *P. hayi*, *P. hayi* with Controls vs. *P. hayi* with *G. affinis*. Both *G. affinis* ($t = 0.58$, $df = 8$, p-value = 0.5747) and *P. hayi* ($t = 1.09$, $df = 8$, p-value = 0.3058) treatments were not context dependent, but Controls were context dependent ($t = 3.14$, $df = 8$, p-value = 0.0137) (Fig. 8). There were significantly fewer eggs rafts in Controls when paired with *G. affinis* pools.

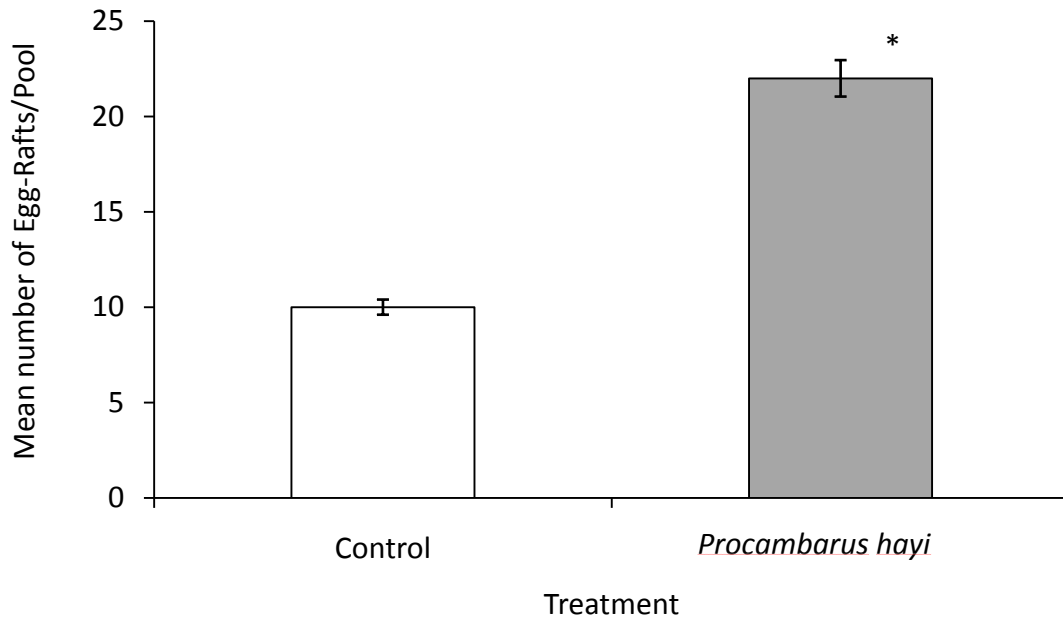


Fig. 5. Mean number (± 1 SE) of egg rafts per pool deposited in Control versus Crayfish-conditioned treatments. White bars represent Controls while gray bars represent Crayfish-conditioned pools. The mean number of egg rafts in Crayfish-conditioned pools was significantly higher than in Control pools.

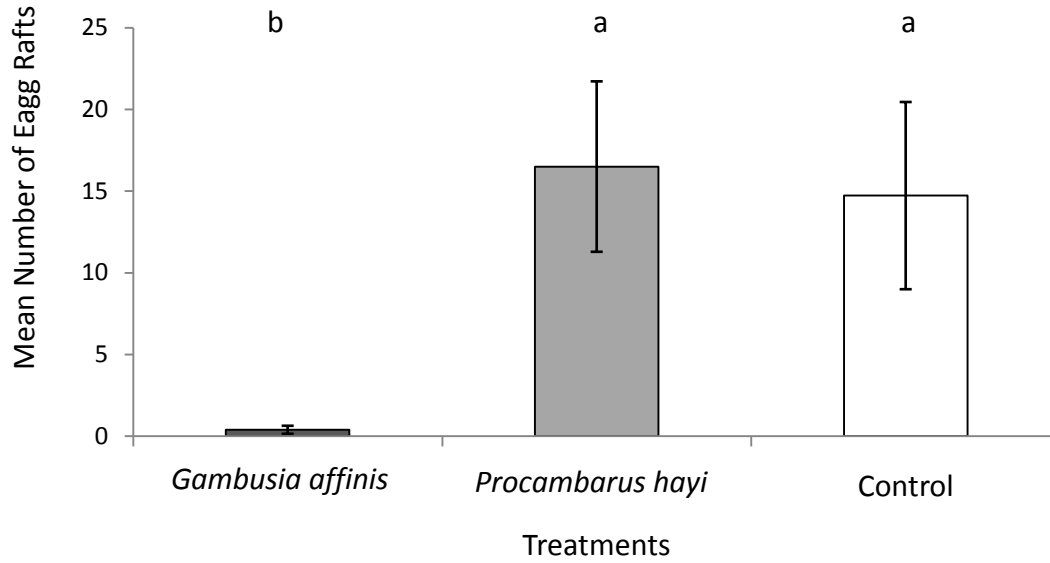


Fig. 6. Mean number ($\text{SE} \pm$) of egg rafts per pool deposited in *Gambusia affinis*, *Procambarus hayi*, and Control pools. Different letters indicate significance ($p < 0.05$) between treatments. The mean number of egg rafts in the *Procambarus hayi* and Control treatments were significantly higher than the *Gambusia affinis* treatment.

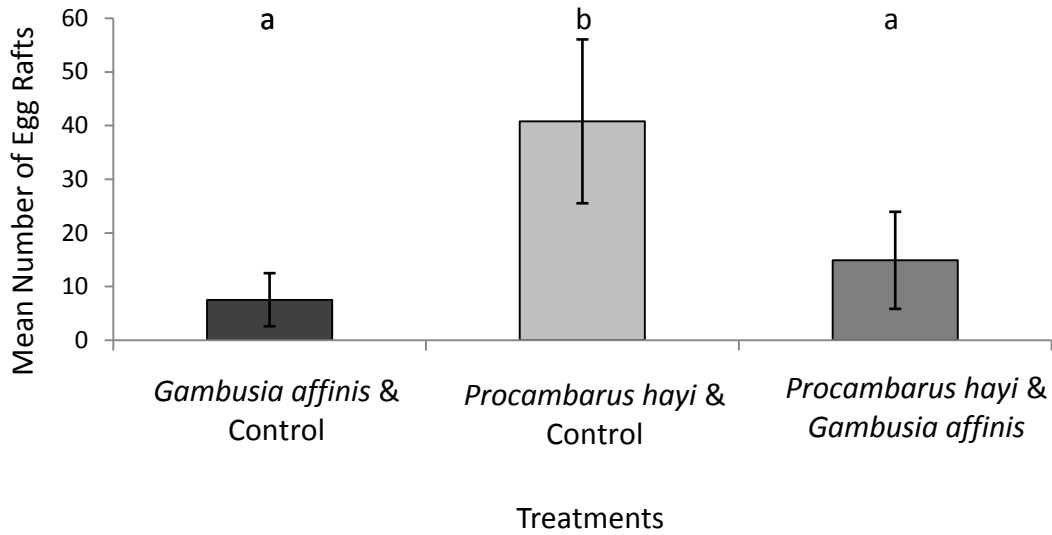


Fig. 7. Mean number ($\pm 1 \text{ SE}$) of egg rafts per pool deposited in paired pools. Different letters indicate significance ($p < 0.05$) between pair types. The mean number of egg rafts collected from *Procambarus hayi*/Control was significantly greater than *Gambusia affinis*/Control and *Procambarus hayi* /*Gambusia affinis* pairs.

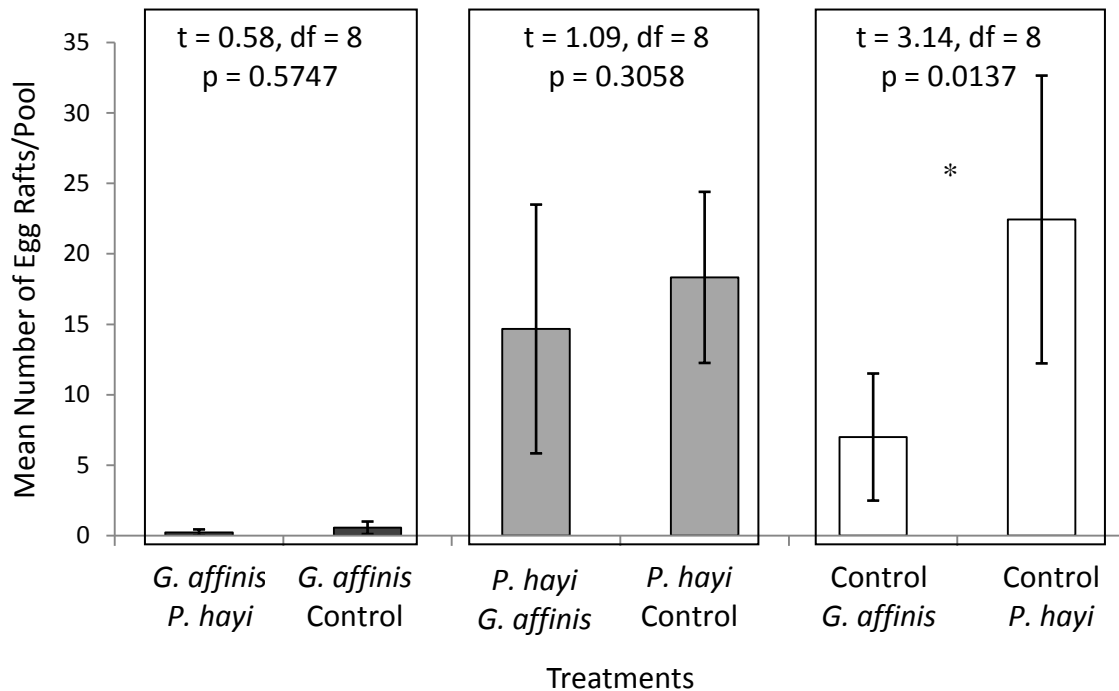


Fig. 8. Mean number (± 1 SE) of egg rafts per pool deposited in all six pool types as defined by pool type (Top label) and the pool type it was paired with (Bottom label). Each box represents the treatments that were analyzed using a paired, two-tailed Student's t-test. The results indicated that *G. affinis* and *P. hayi* treatments were not context dependent, but Controls were context dependent with Control/*G. affinis* pools having significantly fewer egg rafts than Control/*P. hayi* pools. * indicates significance ($p < 0.05$).

II.IV DISCUSSION

Choosing an oviposition site is a critical behavior for mosquitoes and other organisms that do not provide care for their offspring. Determining the mechanisms gravid mosquitoes use to locate a habitat in which to lay their eggs is important since it is the only parental care they provide for their offspring. Selecting an appropriate larval habitat not only ensures the health and survival of the offspring, but also increases the fitness of the adults. Certain mosquito species from the genus *Culex* are particularly selective and are able to detect predator presence via kairomones (Blaustein et al. 2005, Walton 2008, Eveland et al. *submitted*). This mechanism aids female mosquitoes in avoiding high-risk habitats. The focus of this study was to determine how

ovipositing *Culex* mosquitoes react to crayfish-released kairomones, and how individuals responded when presented combinations of positive (*Procambarus hayi*), negative (*Gambusia affinis*), and neutral (well-water) stimuli, and how context affected oviposition behavior.

The results of the first study demonstrate that mosquitoes do have the ability to detect crayfish-released kairomones. Female mosquitoes perceived Crayfish-conditioned pools differently than Control pools, preferring Crayfish-conditioned pools as an oviposition site. The results from the second study provided further evidence that mosquitoes avoid *G. affinis*. In addition, *C. restuans* perception of Control pool quality is context dependent. Spatial contagion of risk seems to be occurring among pools paired with *G. affinis* treatments. Spatial contagion of risk occurs when organisms perceive a suitable patch to have characteristics of a nearby high-risk patch, thus, perceiving the suitable habitat as a low quality habitat (Resetarits et al. 2005). When *G. affinis* pools were paired with *P. hayi* or Control pools, mosquitoes perceived the latter pools as high-risk and unsuitable habitat patches and chooses not to oviposit in those treatments or reduce oviposition activity at those pools more than they would if the pools were not paired with *G. affinis*. This effect of spatial contagion of risk has also been demonstrated in ovipositing treefrogs (Resetarits et al. 2005) and aquatic beetles (Resetarits and Binckley 2009). The highest number of egg rafts was found in pairs of Control and *P. hayi* pools. *Procambarus hayi* treatments received slightly more egg rafts than the Controls, but the difference was not statistically different. These results did not supported the results from the first experiment, where I found significantly more egg rafts laid in Crayfish-conditioned pools than Controls. The only difference between the two was that the prior experiment used only chemical cues and the second study had one crayfish present in the experimental pools. It is possible that ovipositing

mosquitoes were disturbed by crayfish movement, perceiving the habitat less suitable than if only chemical cues were present.

There are numerous reasons why ovipositing female mosquitoes may have perceived habitats with crayfish as more suitable than controls, even though crayfish consume mosquito eggs and larvae. First, crayfish may indicate habitats with high nutrition which is beneficial for mosquito larvae. Crayfish shred the food they consume which releases organic material into the water which would provide nutrients for mosquito larvae thus attracting ovipositing mosquitoes. In addition, crayfish may produce chemicals or bacteria that are favored by female mosquitoes, or the presence of crayfish may indicate habitats that are rich in organic material leading to higher levels of bacteria. Bacteria have been found to be an attractant to gravid mosquitoes (Maw 1970, Suleman and Shirin 1981), which is adaptive since bacteria stimulate larval growth and development (Hazard et al 1967). Although, studies have found that mosquito species vary in their response to different bacteria (Rockett 1987, Hasselschwert and Rockett 1988, reviewed in Bently and Day 1989, Lindh et al 2008) and thus, conclusions should not be drawn for all mosquitoes.

In addition, *P. hayi* are a large crayfish species, and all individuals used in the experiments were adults. Crayfish age and size may have impacted how *C. restuans* responded to crayfish- released kairomones. Adult and juvenile crayfish usually have different diets, with adults feeding more on detritus and vegetation and juveniles being more carnivorous (Butler and Stein 1985, Goddard 1988). In addition, adult crayfish have been found to inhabit deeper water while juveniles occupy shallower water (Butler and Stein 1985, Rabeni 1985, Creed 1994). Thus, since mosquito larvae are found at the water surface, juvenile crayfish may have more access to and consume more larvae than adult crayfish. Developing anti-predator behaviors is costly;

therefore, *C. restuans* may not have developed this behavior if they are not regularly threatened by the crayfish. In order to better understand the results of the two experiments, more field studies should be conducted with different crayfish species at various ages, and testing for the bacteria in the water when crayfish are present. These experiments could give us more insight into why gravid *C. restuans* did not avoid laying eggs in habitats with *P. hayi*.

The results from this study provide information about mosquito oviposition and may provide information about how to examine oviposition in other organisms that oviposit in temporary aquatic systems. It is clear that not all potential predators are perceived equally. This may depend on the species of predator, their location, their diet, life stage, or even the number of individuals present and whether they are physically present or just lingering kairomones. In addition, spatial contagion may occur when predators are in close proximity to quality habitats, since ovipositing females can perceive high quality habitats as low quality when they are in the proximity of high-risk habitats (Resetarits et al. 2005). Continuing research on oviposition behavior in mosquitoes and other animals that oviposit in aquatic systems will help researchers better understand detection mechanisms being used, species interactions, and community structure.

CONCLUSIONS

I examined oviposition site selection in *Culex* mosquitoes and how they respond to the presence of predators that prey on their eggs and larvae. The factors mosquitoes use to select an adequate oviposition site vary from species to species. Indeed, even congeners may vary in how they respond to the presence of predators. Determining the mechanisms gravid mosquitoes use to locate a habitat in which to lay their eggs is important since it is the only parental care they provide for their offspring. Selecting an appropriate larval habitat not only ensures the health and survival of the offspring, but also increases the fitness of the adults.

My studies provide evidence that *C. restuans* respond to and avoid kairomones released by *G. affinis*, but not *L. cyanellus*. This means gravid mosquitoes can spend less time and energy searching for quality habitats to lay their eggs since visual and tactile cues are not necessary. Thus, females would have more time for feeding and predator avoidance. In addition, mosquitoes from the same genus, with very similar ecological niches respond differently to the presence of fish-released kairomones. In this study, *C. p×q* did not respond to the presence of only fish-released kairomones, but Resetarits and Silberbush (*in review B*) discovered that *C. p×q* are deterred by *G. affinis* when there is actually an individual present. The results of the two studies suggest that *C. p×q* may use more than one mechanism (Chapter I). These differences demonstrate that it cannot be assumed that gravid mosquitoes will respond the same way and use the same cues to locate an oviposition site. *Culex restuans* responded differently to *P. hayi* - kairomones, *P. hayi*, and *G. affinis*. Both *G. affinis* and *P. hayi* prey on mosquito larvae, but ovipositing *C. restuans* were only deterred by *G. affinis*. *Culex restuans* may not be deterred by

P. hayi if possible benefits outweigh the cost. For example, crayfish presence may indicate water with high organic material and nutrients, which is beneficial to mosquito larvae. Nutrients alone may outweigh the cost of potential predation of larvae by crayfish. In addition, adult *P. hayi* may not readily consume mosquito larvae in natural habitats. Therefore, *C. restuans* may not be detecting crayfish-released kairomones as being dangerous. Since significantly more egg rafts were deposited in Crayfish-conditioned water than in Controls, crayfish presence may be indicating higher quality habitats compared to habitats without crayfish. High quality habitats could be those with increased nutrients, or other factors that are beneficial to mosquito larvae growth, development, and survival. Furthermore, female mosquitoes seem to perceive perfectly good habitats as low-quality when they are in close proximity (3 m) to mesocosms with *G. affinis* illustrating spatial contagion of risk. This is indicated by the fact that the number of egg rafts deposited in Control and *P. hayi* pools paired with *G. affinis* pools were drastically lower than when Control and *P. hayi* pools were paired together (Chapter II).

Oviposition site selection is a non-random reproductive behavior where individuals actively assess habitats and choose where to lay their eggs (Rausher 1983, Singer 1984). The results from my research indicate that life history characteristics of the model organism, different predator presence, and spatial dynamics all play key parts in selecting an oviposition site.

Determining the mechanisms organisms use to assess and choose oviposition sites will assist in better understanding species distribution, species interactions, and community structure (Resetarits and Wilbur 1989).

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Publications

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